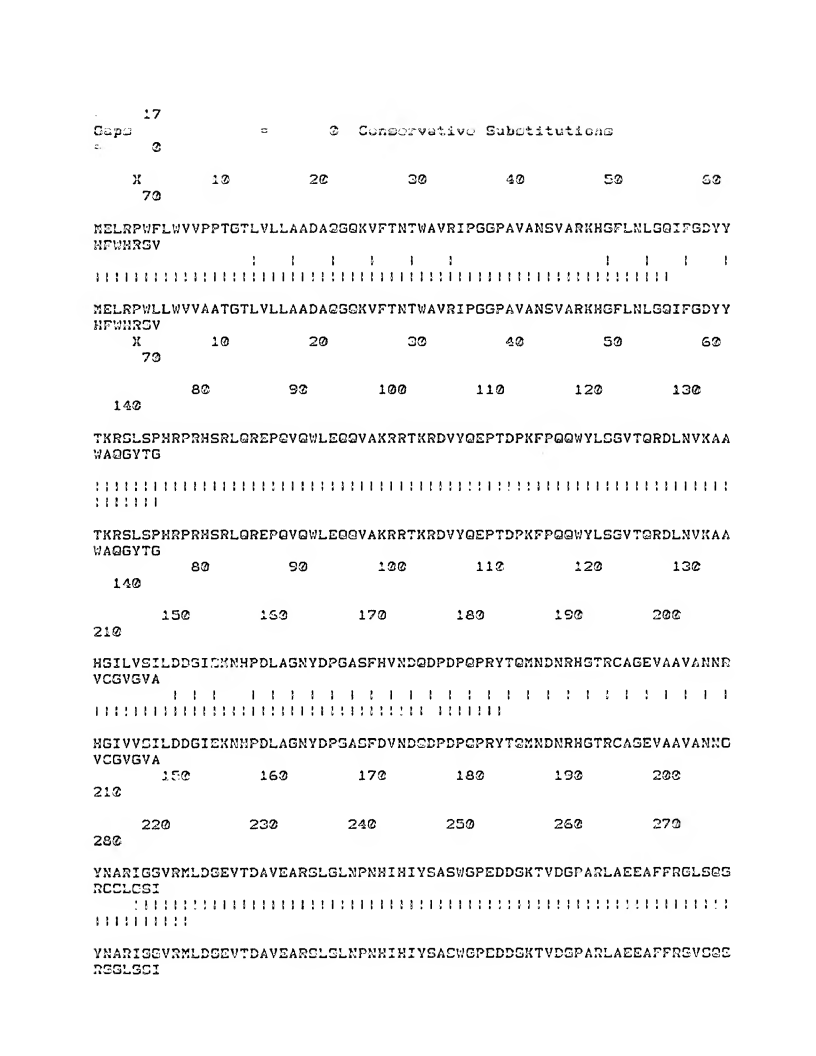
```
FT   Active site      104
FT   Active site      205
FT   /label= oxyanion hole
FT   Active site      358
FT   Cleavage site    102..107
FT   /label= triplet of cleavage sites
FT   Cleavage site    410..412
FT   Cleavage site    440..442
FT   Disulfide bond   211..330
FT   Disulfide bond   303..333
FT   Disulfide bond   305..332
FT   Region           175..184
FT   /label= Ca binding site - external loop
FT   Region           205..212
FT   /label= Ca binding site - external loop
PN   WO9106314-A.
PD   16-MAY-1991.
PF   12-OCT-1990; NL-010151.
PR   25-OCT-1989; NL-000265l.
PR   18-APR-1990; NL-000017.
PA   (NDTH ) NBT HOLLAND BIOTECH.
PI   Vandoven W, Van Den Ouweland AM, Vanduijnhoven JL, Roebroek
AJM;
PI   Koning PN;
DR   WPI; 91-160056/22.
PT     Pharmaceutical compsn. with endo-proteolytic activity -
comprises
PT     furin or its fragment, and is used to prevent obstruction
of
PT   vital organs.
PS   Disclosure; Fig 1; 29pp; English.
CC     The sequence is encoded by the fur gene located in the
genome
CC     upstream of the human fes/fps protooncogene (Van den
Ouweland et
CC     al., Nucl. Acids Res. 17, 1989, 7101-7102). Furin is a
restriction
CC     endopeptidase which processes precursors of polypeptide
hormones,
CC     growth factors, toxins, etc. It can be used to treat
diseases
CC     associated with inadequate processing, or to clear
deposition of
CC     substrate proteins (to alleviate obstruction of vital
organs).
SQ   Sequence   794 AA;
SQ   70 A; 46 R; 33 N; 41 D; 0  B; 22 C; 41 Q; 43 E; 0  Z; 57 G;
26 H;
SQ   25 I; 66 L; 24 K; 8  M; 21 F; 48 P; 61 S; 55 T; 17 W; 22 V;
58 V;

Initial Score      =      777   Optimized Score    =      777
Significance = 58.26
Residue Identity =    97%  Matches          =    777  Mismatches
```


17
Gaps = 0 Conservative Substitutions
0
X 10 20 30 40 50 60
70
MELRPWFLWVVPPTGTLVLLAADAQSQKVFTNTWAVRIPGGPAVANSVARKHGFLNLSQIFGDYY
NFWHRGV
MELRPWLLWVVAATGTLVLLAADAQSQKVFTNTWAVRIPGGPAVANSVARKHGFLNLSQIFGDYY
NFWHRGV
X 10 20 30 40 50 60
70
80 90 100 110 120 130
140
TKRSLSPHRPRHSRLQREPQVQWLEQQVAKRRTKRDVYQEPTDPKFPQQWYLSGVTQRDLNVKAA
WAQGYTG
TKRSLSPHRPRHSRLQREPQVQWLEQQVAKRRTKRDVYQEPTDPKFPQQWYLSGVTQRDLNVKAA
WAQGYTG
80 90 100 110 120 130
140
150 160 170 180 190 200
210
HGILVSILDDGIEKNHPDLAGNYDPGASFHVNDQDPDPQPRYTQMNDNRHGTRCAGEVAAVANNG
VCGVGVA
HGIVVSILDDGIEKNHPDLAGNYDPGASFDVNDQDPDPQPRYTQMNDNRHGTRCAGEVAAVANNG
VCGVGVA
150 160 170 180 190 200
210
220 230 240 250 260 270
280
YNARIGSVRMLDGEVTDAVEARSLGLNPNHIHIYSASWGPEDDGKTVDGPARLAEEAFFRGLSQG
RGGLGSI
YNARIGSVRMLDGEVTDAVEARSLGLNPNHIHIYSASWGPEDDGKTVDGPARLAEEAFFRSVSQG
RGGLGSI

```
ID   P70050 standard; protein; 490 AA.
AC   P70050;
DT   27-JAN-1991  (first entry)
DE   Partial sequence of large open reading frame in 3.1 kb fur
DE   cDNA sequence.
KW   Furin; fur gene; tumor diagnosis.
OS   Homo sapiens, Felis catus.
FH   Key             Location/Qualifiers
FT   Domain          201..410
FT   /label=cysteine-rich region
FT   Domain          410..430
FT   /label=transmembrane domain
FT   Domain          430..450
FT   /label=transmembrane domain
PN   EP-245709-A.
PD   25-NOV-1987.
PF   19-MAY-1987; 200940.
PR   20-MAY-1986; NL-001271.
PA   (UYKA-) Katholieke Univ.
PI   Van de Ven WJM, Roebroek AJM, Schalken JA;
DR   WPI; 87-328946/47.
PT   Recombinant DNA containing the fur gene - used for
producing furin
PT   protein and antibodies and as a diagnostic aid in the
detection of
PT   tumours.
PS   Disclosure; Fig 9A; 24pp; English.
CC   The sequence is the partial amino acid sequence of the
CC   fur gene open reading frame.  The fur gene encoding furin
CC   is in the human and cat genomes directly upstream of the
CC   fes/fps proto-oncogene.  Furin is strongly expressed in
CC   specific types of tumors and labelled RNA or DNA probes of
CC   the fur gene and antibodies against furin can be used for
CC   diagnostic purposes.  (See also N70060-62).
SQ   Sequence   490 AA;
SQ   42 A; 24 R; 18 N; 22 D; 0  B; 21 C; 24 Q; 32 E; 0  Z; 34 G;
16 H;
SQ   15 I; 47 L; 15 K; 5  M; 12 F; 31 P; 47 S; 42 T; 8  W; 13 Y;
31 V;

Initial Score       =       487   Optimized Score   =       487
Significance = 36.38
Residue Identity =     97%  Matches           =     487  Mismatches
=     12
Gaps             =       0  Conservative Substitutions
=       0

      290       X 300         310         320         330         340
350

GSIFVWASGNGGREHDSCNCDGYTNSIYTLSISSATQFGNVPWYSEACSSTLATTYSSGNQNEKQ
IVTTDLR

:::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
```

APS 07/02/1092

US PAT NO: 4,992,373 [IMAGE AVAILABLE] L6: 1 of 5

ABSTRACT:
A method for the direct recombinant production of activated protein C is described. DNA compounds, vectors, and transformants useful in the method are also disclosed. The method involves transformation and culture of a host cell with a recombinant DNA vector that encodes a protein C molecule in which the activation peptide is replaced with a cleavage sequence for a cell associated protease.

1. 4,992,373, Feb. 12, 1991, Vectors and compounds for direct expression of activated human protein C; Nils U. Bang, et al., 435/226, 172.3, 240.1, 240.2, 254, 320.1; 536/27 [IMAGE AVAILABLE]

US PAT NO: 4,959,318 [IMAGE AVAILABLE] L6: 2 of 5

ABSTRACT:
Genomic and cDNA sequences coding for a protein having substantially the same biological activity as human protein C and recombinant transfer vectors comprising these sequences are disclosed. Methods are disclosed for producing a protein which has substantially the same biological activity as human protein C. The protein, which may be in the form of activated protein C, is produced by mammalian host cells transfected with a plasmid capable of integration in mammalian host cell DNA. The plasmid includes a promoter followed downstream by a nucleotide sequence which encodes a protein having substantially the same structure and/or activity as human protein C, the nucleotide sequence being followed downstream by a polyadenylation signal.

2. 4,959,318, Sep. 25, 1990, Expression of protein C; Donald C. Foster, et al., 435/172.3, 226, 240.25, 320.1, 849; 536/27; 935/14, 29, 32, 48 [IMAGE AVAILABLE]

US PAT NO: 4,784,950 L6: 3 of 5

ABSTRACT:
Methods are disclosed for producing proteins having biological activity for **blood** **coagulation** mediated by Factor VIIa. The proteins are produced by mammalian host cells which have been stably transfected with a DNA construct containing a nucleotide sequence which codes at least partially for either Factor VII. The nucleotide sequence comprises a first nucleotide sequence encoding a calcium binding domain, joined to a second nucleotide sequence positioned downstream of the first sequence. In particular, the first nucleotide sequence may be derived from a genomic clone or cDNA clone of Factor VII. The second sequence encodes a catalytic domain for the serine protease activity of Factor VIIA. The joined sequences code for proteins having substantially the same biological activity for **blood** **coagulation** as Factor VIIa.

3. 4,784,950, Nov. 15, 1988, Expression of factor VII activity in mammalian cells; Frederick S. Hagen, et al., 435/69.6, 172.3, 219, 240.2, 320.1; 530/384; 536/27; 930/10; 935/11, 32, 48, 60, 70

US PAT NO: 4,775,624 [IMAGE AVAILABLE] L6: 4 of 5

ABSTRACT:
The present invention comprises novel DNA compounds which encode human protein C activity. A variety of eukaryotic and prokaryotic recombinant DNA expression vectors have been constructed that comprise the novel protein C activity-encoding DNA and drive expression of protein C activity when transformed into an appropriate host cell. The novel expression vectors can be used to produce protein C derivatives, such as non-carboxylated, non-glycosylated, or non-hydroxylated protein C, and to produce protein C precursors, such as reagent or exogen protein C, and

to produce sub-fragments of protein C, such as active or inactive light and heavy chain. The recombinant-produced protein C activity is useful in the treatment and prevention of a variety of vascular disorders.

4. 4,775,624, Oct. 4, 1988, Vectors and compounds for expression of human protein C; Nils U. Bang, et al., 435/226, 69.6, 172.3, 240.25, 252.33, 320.1, 832, 849, 886; 536/27; 930/10, 240; 935/14, 29, 32 [IMAGE AVAILABLE]

US PAT NO: 4,770,999 L6: 5 of 5

ABSTRACT:

High yields of active **Factor** **IX** are produced by culturing a CHO cell line transfected with chromosomally-integrated **Factor** **IX** cDNA in medium to which vitamin K is added.

5. 4,770,999, Sep. 13, 1988, High yield production of active **Factor** **IX**; Randal J. Kaufman, et al., 435/69.6, 91, 172.3, 240.2, 317.1; 536/27; 935/14, 55, 62, 70

=> d his

(FILE 'USPAT' ENTERED AT 11:14:10 ON 08 JAN 92)

L1 9 S GAMMA-CARBOXYLATION
L2 928 S BLOOD(W)COAGULATION
L3 928 S BLOOD-COAGULATION
L4 6 S L1 AND L2
L5 185 S FACTOR-IX
L6 5 S L4 AND L5

=> d kwic l6 1

US PAT NO: 4,992,373 [IMAGE AVAILABLE] L6: 1 of 5

SUMMARY:

BSUM(4)

The Role of Protein C in the Regulation of **Blood** **Coagulation**

SUMMARY:

BSUM(7)

To understand how activated protein C down-regulates **blood** **coagulation**, the following brief description of the coagulation enzyme system is provided. The coagulation system is best looked at as a.

SUMMARY:

BSUM(12)

In . . . suffer severe, recurrent thromboembolic episodes. It is well established clinically that plasma protein concentrates designed to treat Hemophilia B or **factor** **IX** deficiency, which contain protein C as an impurity, are effective in the prevention and treatment of intravascular clotting in heterozygous. . .

SUMMARY:

BSUM(19)

Nascent . . . of nascent human protein C encode the signal peptide and propeptide of human protein C, important for direction secretion and

gamma.-**carboxylation** of protein C.

SUMMARY:

BSUM(37)

.**gamma**.-**carboxylation**--a reaction which adds a carboxyl group to glutamic acids at the .gamma.-carbon.

SUMMARY:

BSUM(38)

.gamma.-carboxylated protein--a protein in which some glutamic acids residues have undergone .**gamma**.-**carboxylation**.

SUMMARY:

BSUM(41)

Nascent protein--the polypeptide produced upon translation of a mRNA transcript, prior to any post-translational modifications. However, post-translational modifications such as .**gamma**.-**carboxylation** of glutamic acid residues and hydroxylation of aspartic acid residues may occur before a protein is fully translated from an. . .

DETDESC:

DETD(14)

The . . . of the pre-propeptide of a .gamma.-carboxylated protein. Examples of such .gamma.-carboxylated proteins include, but are not limited to, factor VII, **factor** **IX**, factor X, prothrombin, protein S, protein Z, and, most preferably, protein C.

DETDESC:

DETD(15)

The . . . C, are responsible for calcium-binding activity of these proteins. The calcium-binding domains of these plasma proteins, such as factor VII, **factor** **IX**, factor X, prothrombin, and protein S, are interchangeable (see European Patent Publication No. 0215548A1, at pages 12 and 13) and. . .

DETDESC:

DETD(20)

. . .
proteases
so as to generate the removal of the propeptide from
secreted proteins include:

glucagon	MLVEGSW@
protein C	@VLRIRKR
factor **IX**	KILNRPKR
factor X	NILARVTR
tissue plasminogen activator	ARFRRGAR

Cleavage sequences recognized by cell associated proteases so as to. . .

DETDESC:

DETD(28)

As . . . signal peptide and proteolytic cleavage site on the compounds of the invention and provide the post-translational modifications, such as glycosylation, .**gamma**.-**carboxylation**, and beta.-hydroxylation, as are observed in human protein C present in blood plasma. A wide variety of vectors, discussed below,. . .

=> d kwic 16 1

US PAT NO: 4,959,318 [IMAGE AVAILABLE] LG: 2 of 5

SUMMARY:

BSUM(4)

Protein C is a zymogen, or precursor, of a serine protease which plays an important role in the regulation of **blood** **coagulation** and generation of fibrinolytic activity in vivo. It is synthesized in the liver as a single-chain polypeptide which undergoes considerable. . .

SUMMARY:

BSUM(6)

In contrast to the coagulation-promoting action of other vitamin K-dependent plasma proteins, such as factor VII, **factor** **IX**, and factor X, activated protein C acts as a regulator of the coagulation process through the inactivation of factor Va. . .

SUMMARY:

BSUM(15)

The proteins described within the present invention may be used as active therapeutic substances, including use in the regulation of **blood** **coagulation**. Further, these proteins may be combined with a physiologically acceptable carrier and/or diluent to provide suitable pharmaceutical compositions.

DETDESC:

DETD(9)

Given the fact that the activity of protein C is dependent upon post-translational modifications involving the **gamma** **carboxylation** of specific glutamic acid residues and cleavage to the two-chain form, and may also be dependent upon the hydroxylation of. .

DETDESC:

DETD(12)

The . . . proteins show considerable overall structural homology to each other and to other vitamin K-dependent plasma proteins, including prothrombin, factor VII, **factor** **IX**, and factor X. Similarities include the presence of the Gla residues in the light chain and the active site serine. . .

DETDESC:

DETD(26)

The . . . as described in U.S. patent application Ser. No. 724,311, filed Apr. 17, 1985, the amino-terminal portion (calcium binding domain) of **factor** **IX** may be joined to factor VII at amino acid 26 to

produce a protein having the activity of factor VII. Factor VII, **factor** **IX**, factor X, prothrombin, and protein S share this amino-terminal sequence homology with protein C. Consequently, a cloned sequence comprising the. . .

DETDESC:

DETD(27)

When . . . according to the present invention. The pre-pro peptide may be that of protein C or another secreted protein, such as **factor** **IX**, factor VII, or prothrombin.

DETDESC:

DETD(89)

To assess the extent of **gamma**-**carboxylation** of the recombinant protein, samples of the culture media were subjected to barium citrate precipitation, a process which selectively precipitates. . .

=> d kwic 16 3

US PAT NO: 4,784,950 L6: 3 of 5

ABSTRACT:

Methods are disclosed for producing proteins having biological activity for **blood** **coagulation** mediated by Factor VIIa. The proteins are produced by mammalian host cells which have been stably transfected with a DNA. . . the serine protease activity of Factor VIIA. The joined sequences code for proteins having substantially the same biological activity for **blood** **coagulation** as Factor VIIa.

SUMMARY:

BSUM(2)

The present invention relates to **blood** **coagulation** factors in general, and more specifically, to the expression of proteins having biological activity for **blood** **coagulation**.

SUMMARY:

BSUM(5)

Blood **coagulation** is a process consisting of a complex interaction of various blood components or factors which eventually gives rise to a. . .

SUMMARY:

BSUM(6)

There . . . formation through utilization of factors present only in plasma. An intermediate event in the intrinsic pathway is the activation of **Factor** **IX** to Factor IXa, a reaction catalyzed by Factor XIa and calcium ions. Factor IXa then participates in the activation of. . components present in tissue extracts. Factor VII, one of the proenzymes referred to above, participates in the extrinsic pathway of **blood** **coagulation** by converting (upon its activation to VIIa) Factor X to Xa in the presence of tissue factor and calcium ions.. . . pathway as well (Zur and Nemerson, J. Biol. Chem. 253: 2208-2200, 1978) by playing a role in the activation of **Factor** **IX**.

SUMMARY:

SUM(8)

Factor **IX** circulates in the blood as a single-chain precursor of molecular weight 57,000 and is converted to an active serine protease. .

SUMMARY:

BSUM(10)

Therapeutic . . . Factor VII exist in the treatment of individuals inhibiting a deficiency in Factor VII, as well as Factor VIII and **Factor** **IX** deficient populations, and individuals with Von Willebrand's disease. More specifically, individuals receiving Factors VIII and IX in replacement therapy frequently. . .

SUMMARY:

BSUM(12)

Consequently, . . . a need in the art for a method of producing relatively large quantities of pure preparations of Factors VIIa and **Factor** **IX**. The present invention fulfills this need through the use of recombinant DNA technology, successfully eliminating the problem of viral contamination. . . and, at the same time, providing a consistent and homogeneous source of active Factor VIIa to treat Factor VIII and **Factor** **IX** deficient patients and individuals with Von Willebrand's disease, as well as providing a source of purified **Factor** **IX** for use in replacement therapy.

SUMMARY:

BSUM(14)

Briefly . . . of Factor VIIa. The joined sequences code for a protein which upon activation has substantially the same biological activity for **blood** **coagulation** as Factor VIIa. The first nucleotide sequence may be substantially that of a gene encoding Factor VII, **Factor** **IX**, Factor X, Protein C, prothrombin, or Protein S. Further, the first nucleotide sequence may also encode a leader peptide corresponding.

SUMMARY:

BSUM(15)

In . . . include a double-stranded oligonucleotide. A particularly preferred first nucleotide sequence is that encoding the leader peptide and amino-terminal portion of **Factor** **IX**.

SUMMARY:

BSUM(16)

In . . . of Factor VIIa. The joined sequences code for a protein which upon activation has substantially the same biological activity for **blood** **coagulation** as Factor VIIa. The nucleotide sequence is then followed downstream by a polyadenylation signal.

SUMMARY:

BSUM(17)

Similar . . . RNA splice sites, the RNA splice sites being followed downstream by a nucleotide sequence which codes at least partially for **Factor** **IX**. The nucleotide sequence comprises a first [illegible]

...sequence which encodes a calcium binding domain joined to a second ...nucleotide sequence. . . positioned downstream of the first sequence. The second nucleotide sequence encodes a catalytic domain for the serine protease activity of **Factor** **IX**. The joined sequences code for a protein having substantially the same biological activity for **blood** **coagulation** as **Factor** **IX**. The nucleotide sequence is then followed downstream by a polyadenylation signal.

SUMMARY:

SCUM(18)

A . . . of Factor VIIa. The joined sequences code for a protein which, upon activation, has substantially the same biological activity for **blood** **coagulation** as Factor VIIa.

SUMMARY:

SCUM(19)

An . . . aspect of the invention discloses mammalian cells stably transfected to produce a protein having substantially the same biological activity as **Factor** **IX**. The cells are transfected with a DNA construct containing a nucleotide sequence which codes at least partially for **Factor** **IX**. The nucleotide sequence comprises a first nucleotide sequence which encodes a calcium binding domain joined to a second nucleotide sequence. . . positioned downstream of the first sequence. The second nucleotide sequence encodes a catalytic domain for the serine protease activity of **Factor** **IX**. The joined sequences code for a protein having substantially the same biological activity for **blood** **coagulation** as **Factor** **IX**.

SUMMARY:

SCUM(20)

The present invention further provides for a method of producing a protein having biological activity for **blood** **coagulation** mediated by Factor VIIa through establishing a mammalian host cell which contains a DNA construct containing a nucleotide sequence which. . . of Factor VIIa. The joined sequences code for a protein which, upon activation, has substantially the same biological activity for **blood** **coagulation** as Factor VIIa. Subsequently, the mammalian host is grown in an appropriate medium and the protein product encoded by the. . .

SUMMARY:

SCUM(21)

Still a further aspect of the present invention discloses a method of producing a protein having biological activity for **blood** **coagulation** mediated by **Factor** **IX**. The method comprises establishing a mammalian host cell which contains a DNA construct containing a nucleotide sequence which codes at least partially for **Factor** **IX**. The nucleotide sequence comprises a first nucleotide sequence which encodes a calcium binding domain joined to a second nucleotide sequence. . . positioned downstream of the first sequence. The second nucleotide sequence encodes a catalytic domain for the serine protease activity of **Factor** **IX**. The joined sequences code for a protein having substantially the same biological activity for **blood** **coagulation** as **Factor** **IX**. The mammalian host cell is subsequently grown in an appropriate medium and the protein product encoded by the mammalian host. . .

SUMMARY:

BSUM(24)

A method for providing a protein having biological activity for **blood** **coagulation** mediated by Factor VIIa through establishing a formation host cell that contains a DNA construct or vector that ... .

DRAWING DESC:

DRWD(6)

FIG. 3 illustrates the joining of **Factor** **IX** leader sequences to a sequence encoding a gamma-carboxy calcium binding domain.

DRAWING DESC:

DRWD(7)

FIG. 4 illustrates the joining of the **Factor** **IX**-gamma hexapeptide sequence hybrid to a partial Factor VII cDNA to produce an in-frame coding sequence.

DRAWING DESC:

DRWD(8)

FIG. 5 illustrates the construction of a plasmid containing a coding sequence for a **Factor** **IX**/Factor VII fusion protein.

DRAWING DESC:

DRWD(10)

FIG. 7 illustrates the nucleotide sequence of a **Factor** **IX**/Factor VII cDNA fusion.

DETDESC:

DETD(10)

For Factor VIIa, biological activity is characterized by the mediation of **blood** **coagulation** through the extrinsic pathway. Factor VIIa activates Factor X to Factor Xa, which in turn converts prothrombin to thrombin, thereby . . . of a fibrin clot. Because the activation of Factor X is common to both the extrinsic and intrinsic pathways of **blood** **coagulation**, Factor VIIa may be used to treat individuals severely deficient in the activities of **Factor** **IX**, Factor VIII or Von Willebrand Factor.

DETDESC:

DETD(11)

The biological activity of **Factor** **IX** is characterized by the mediation of **blood** **coagulation** through the intrinsic pathway. **Factor** **IX** is activated to Factor IXa by Factor XIa. Factor IXa then activates Factor X to Factor Xa in the presence . . .

DETDESC:

DETD(12)

At . . . at a concentration of approximately 300 nanograms per liter of blood. In addition, it is difficult to separate from prothrombin, **Factor** **IX** and Factor X and is susceptible to proteolytic attack [illegible]

human Factor VII. . .

DETDESC:

DETD(14)

Given the fact that the activities of Factors VII and IX are dependent upon post-translational modifications involving the **gamma** **carboxylation** of specific glutamic acid residues, and may also be dependent upon the hydroxylation of a specific aspartic acid residue, it.

DETDESC:

DETD(15)

Accordingly, the present invention provides a method of producing a protein having biological activity for **blood** **coagulation** mediated by Factor VIIa using stably transfected mammalian cells. In addition, the present invention also provides a method of producing a protein having biological activity for **blood** **coagulation** mediated by **Factor** **IX**.

DETDESC:

DETD(16)

As . . . homologous in both amino acid sequence and in biological function (FIG. 2a). Further, the carboxy-terminal portions of Factor VII, prothrombin, **Factor** **IX**, Factor X, and Protein C determine their specific serine protease functions.

DETDESC:

DETD(17)

Factor . . . McMullen (ibid). Due to these difficulties, Factor VII has been poorly characterized, compared to other more abundant components of the **blood** **coagulation** system. Indeed, the work of Kisiel and McMullen (ibid) yielded sequence information for only 10 residues of each chain of. . .

DETDESC:

DETD(19)

In comparison to Factor VII, **Factor** **IX** is a relatively abundant protein and the sequence of a cDNA clone of the human **Factor** **IX** gene is known (Kurachi and Davie, Proc. Natl. Acad. Sci. USA 79: 6461-6464, 1982; and Anson et al., EMBO J. 3: 1053-1060, 1984). The structure of the **Factor** **IX** gene has been characterized and the amino acid sequence of the protein has been determined on the basis of the known nucleotide sequence. Some protein sequence data have also been published for human and bovine **Factor** **IX** and the sequences analyzed (DiScipio et al., ibid). The amino terminal portion of the protein contains 12 glutamic acid residues. . . that are converted to beta.-carboxyglutamic acid (Gla) residues in the mature protein. The cleavage sites involved in the activation of **Factor** **IX** have also been identified (Kurachi and Davie, ibid). A sequence at the 3' end of the **Factor** **IX** cDNA clone codes for a signal peptide which is typical of those found in most secreted proteins (Kurachi and Davie, ibid). The expression of the **Factor** **IX** gene through recombinant DNA methods has not been previously reported.

DETDESC:

DETD(20)

Because . . . a partial cDNA clone for Factor VII is joined to a fragment encoding the leader peptide and 5' portion of **Factor** **IX**. This approach is based on the observation that the amino-terminal portions of the two molecules are responsible for the calcium binding activities of the respective proteins and the discovery that the calcium binding activity of **Factor** **IX** can substitute for that of Factor VII. The resultant polypeptide retains the biological activity of authentic Factor VII because the. . . approach involved joining the partial cDNA clone to hybrid coding sequences comprising a cDNA fragment encoding the leader peptide of **Factor** **IX** and a synthetic gene segment encoding a consensus calcium binding domain or a predicted amino terminal sequence for Factor VII.. . .

DETDESC:

DETD(24)

The leader encoded by .lambda.VII2463 is exceptionally long (60 amino acids) and has a very different hydrophobicity profile when compared with **Factor** **IX**, protein C and prothrombin. This leader contains two sets, at positions -52 and -25. Initiation most likely begins at the. . exon-like region in the genomic clone, results in a 38 amino acid leader with a hydrophobicity pattern more analogous to **Factor** **IX**, protein C, and prothrombin.

DETDESC:

DETD(27)

The . . . downstream from the promoter and upstream from the insertion site for a gene encoding a protein having biological activity for **blood** **coagulation**. Preferred RNA splice site sequences may be obtained from adenovirus and/or immunoglobulin genes. Also contained in the expression vectors is. . .

DETDESC:

DETD(29)

Factor VII and **Factor** **IX** produced by the transfected cells may be removed from the cell culture media by adsorption to barium citrate. Spent medium. . .

DETDESC:

DETD(31)

In summary, the present invention provides a method for the production of proteins having the activity of vitamin K-dependent **blood** **coagulation** factors using transfected mammalian cells. Gene sequences encoding the specific serine protease domains of the coagulation factors are isolated from. . . are then joined in an appropriate expression vector so as to encode a protein having the desired biological activity for **blood** **coagulation**. The resulting vector and a plasmid containing a drug resistance marker are co-transfected into appropriate mammalian tissue culture cells. Transfected. . . as G-418. The protein products are then purified from the cell growth media and assayed for biological activity in a **blood** **coagulation** assay and for immunological cross-reactivity using antibodies prepared against authentic human clotting factors.

DETDESC:

DETD(32)

To . . . Example 4 discloses the construction of two hybrid gene segments, each comprising a cDNA fragment encoding the leader peptide of **Factor** **IX** and a synthesized double-stranded fragment encoding a consensus calcium binding domain. The hybrid sequences are then joined to partial cDNA. . . VII. Example 5 describes the construction of a gene sequence encoding a fusion protein comprising the calcium binding domain of **Factor** **IX** and the specific serine protease domain of Factor VII. Example 6 describes the construction of the vector pD2 for use in expressing proteins having biological activity for **blood** **coagulation** in transfected mammalian cells. The gene fusion described in Example 5 is expressed using this vector. Example 7 describes the use of the vector pD2 to express a gene for **Factor** **IX** in a transfected mammalian cell line. Example 8 describes the construction of the vector pM7125, which contains DNA sequences encoding a primary translation product comprising the leader sequence of **Factor** **IX** fused to Factor VII. This vector may be used to produce a protein having the activity of Factor VII in. . .

DETDESC:

DETD(56)

Further . . . at the cleavage site of Factor VII (Kisiel and McMullen, Thrombosis Research 22: 375, 1981). Comparison of this sequence to **Factor** **IX** (Davie et al., ibid) and Factor X (Leytus et al., Proc. Natl. Acad. Sci. U.S.A. 81: 3699-3702, 1984) amino acid. . .

DETDESC:

DETD(61)

Because . . . (Kurachi and Davie, ibid; and Davie et al., ibid), and join this to a portion of the prepro sequence of **Factor** **IX**. The third strategy relies on the functional homology of the amino terminal regions of Factor VII and **Factor** **IX**. A sequence was constructed which comprised the coding regions for the leader and amino-terminal portion of **Factor** **IX**. This was then fused in the proper orientation to the partial Factor VII cDNA.

DETDESC:

DETD(66)

The leader encoded by .lambda.VII2403 is exceptionally long (50 amino acids) and has a very different hydrophobicity profile when compared with **Factor** **IX**, protein C and prothrombin. This leader contains two Meto, at positions -60 and -26. Initiation most likely begins at the. .

DETDESC:

DETD(69)

Factor **IX**-Factor VII Hybrid Genes Containing a Synthesized Coding Sequence

DETDESC:

DETD(94)

A. Construction of a hybrid **Factor** **IX** leader-synthetic Factor VII 5' coding sequence.

DETDESC:

DETD(95)

The . . . Factor VIII coding, this synthetic fragment (the connector sequence) was joined to one of two leader sequences derived from a **Factor** **IX** cDNA clone. This strategy is outlined in FIG. 3.

DETDESC:

DETD(86)

A cDNA coding for human **Factor** **IX** was obtained from a library made with mRNA from human liver (Kurachi and Davie, ibid.). The **Factor** **IX** sequence was isolated from the pBR322 vector by digestion with Pst I and was inserted into the Pst I site. . . . plasmid was designated FIX-pUC13. In order to remove the G-rich region which was present at the 5' end of the **Factor** **IX** insert as a result of cDNA cloning, a synthetic oligonucleotide adapter was substituted for the 5' end of the cloned. . . . overlap, the fragment ends filled in and cut with appropriate restriction endonucleases, and the resulting fragment was joined to the **Factor** **IX** sequence.

DETDESC:

DETD(88)

The modified **Factor** **IX** sequence was then constructed by combining 0.16 pmoles of the synthetic Pst I-Cfo I adapter fragment, 0.14 pmoles of a 1.4 kb Cfo I-Bam HI **Factor** **IX** fragment from FIX-pUC13, and 0.14 pmoles of a 2.7 kb Bam HI-Pst I pUC13 vector fragment in a 20 ul. . .

DETDESC:

DETD(89)

In order to confirm the sequence of the altered region of the **Factor** **IX** portion of the FIX(-G).fvderv.pUC13 construct, dideoxy sequencing directly on the pUC plasmid using the BRL reverse primer was performed using. . .

DETDESC:

DETD(90)

The resulting recombinant plasmid contains three Nae III cleavage sites, the first at position 39 in the **Factor** **IX** sequence (numbering is based on the published sequence of Anson et al. (ibid), beginning at the first ATG), the second. . . site at 130 is a single base pair upstream from the codons for the Lys-Arg processing site of the propro **Factor** **IX** molecule. In the final **Factor** **IX**-Factor VII hybrid constructs, the **Factor** **IX** leader sequence, terminated at position 30 to 130, was joined to a synthetic double-stranded fragment comprising the predicted consensus sequence and the last 3 codons of the **Factor** **IX** leader sequence.

DETDESC:

DETD(92)

The modified **Factor** **IX** fragment was removed from FIX(-G).fvderv.pUC13 as a Hind III-Eco RI fragment. Approximately 20 ug of plasmid was digested with 30. . . at 37.degree. C. overnight. The reaction was terminated by heating at 65.degree. C. for 10 minutes, and the vector and **Factor** **IX** fragments were electrophoresed on a 1% agarose gel and purified by electro-elution. The **Factor** **IX** fragment was precipitated with ethanol, resuspended in buffer containing [illegible] DNase A [illegible] digested with 0.[illegible] of Nae III [illegible] at

37.degree. C. The Hind III-Hae III 29 base pair **Factor** **IN** fragment was isolated from this digest by electrophoresis on a 1.5% agarose gel followed by electroelution. To obtain the Hind II-Hae III 100 base pair **Factor** **IN** fragment, FIN-pUC18 was digested with Eco RI and Hind III and the **Factor** **IN** fragment isolated as above. Approximately 2 ug of this Hind III-Eco RI fragment was digested with 2 units of Hae. . .

DETDESC:

DETD(93)

The final **Factor** **IN**-consensus sequence hybrids were prepared by joining, in a four-part ligation, oligonucleotide pools 1 and 2, **Factor** **IN** Hind III-Hae III (29 or 100 base pairs), and pUC18 Hind III-Eco RI. The resulting plasmids were used to transform. . . Plasmids were screened by digestion of DNA with Eco RI and Hind III. The sequence comprising the 29 base pair **Factor** **IN** sequence joined to the synthetic consensus sequence is hereinafter referred to as mini-FIN-FVII. The plasmid containing this construct was designated pM7292(-C). The sequence comprising the 100 base pair **Factor** **IN** sequence joined to the synthetic consensus sequence is referred to as mini-FIN-FVII. The plasmid containing this construct was designated pM7100(-C).. . .

DETDESC:

DETD(94)

D. Joining **Factor** **IN**-consensus sequence hybrid fragment to Factor VII cDNA clone.

DETDESC:

DETD(95)

The **Factor** **IN**-consensus sequence hybrids (either mini or maxi) were joined to the 5' portion of the Factor VII cDNA and the vector. . The desired fragments were electroeluted, extracted with phenol/CHCl.sub.3 and CHCl.sub.3, and precipitated with ethanol. The three fragments, pUC19/Nbe I-Hind III, **Factor** **IN**-Factor VII (mini or maxi)/Hind III-Eco RI, and 5' Factor VII/Eco RI-Nbe I were then ligated in 20 ul of ligase. . .

DETDESC:

DETD(96)

The . . . generate correct in frame coding sequences. Both mini- and maxi-fusions contain an Eco RI site at the junction between the **Factor** **IN**-consensus sequence hybrid and the Factor VII cDNa which is an artifact of the cDNA cloning process. In addition, the mini-fusion.

DETDESC:

DETD(101)

Construction of **Factor** **IN** Factor VII cDNA fusion

DETDESC:

DETD(102)

The **Factor** **IN**-Factor VII cDNA fusion was prepared using **Factor** **IN** cDNA (plasmid for a human liver cDNA library as

DETDESC:

DETD(103)

The fusion point chosen for the hybrid protein was between amino acid 220 (threonine) of **Factor** **IX** and the first lysine encoded by the Factor VII cDNA sequence. Such a protein would be encoded by a sequence consisting of the first 252 bp of the **Factor** **IX** cDNA sequence and all of the pUCVII2115 Factor VII cDNA sequence except the first two codons. To construct this hybrid sequence, the **Factor** **IX** sequence was first fused to pUCVII2115 using convenient restriction sites. This fusion resulted in the plasmid FIX/VII/12 (described below) which contains the first 210 bp of the **Factor** **IX** cDNA joined to the entire Factor VII cDNA sequence. To achieve the precise junction desired for the hybrids protein, the. . .

DETDESC:

DETD(104)

Joining of the **Factor** **IX** cDNA sequence to Factor VII cDNA sequence was accomplished by ligating a 0.3 kb Hind III-Ahe III fragment of FIX. . .

DETDESC:

DETD(106)

The oligonucleotide-directed mutagenesis procedure was performed on a single-stranded DNA template. Thus, it was necessary to clone the fused **Factor** **IX**/Factor VII sequences into M13mp19. To obtain a conveniently small DNA fragment, a 640 bp Hind III-Xba I fragment was isolated from FIX/VII/12. This fragment contains 310 bp of the 5' end of **Factor** **IX** cDNA and 330 bp of the Factor VII sequence. The vector was prepared by digesting 1 ug of M13mp19 RF. . . was correct. One of the correct clones (#4) was used as a template in oligonucleotide-directed mutagenesis to produce a functional **Factor** **IX**-Factor VII fusion.

DETDESC:

DETD(107)

The oligonucleotide ZC249, a 20-mer consisting of 12 bp of the desired **Factor** **IX** sequence and 10 bp of the desired Factor VII sequence (Table 1) was used as the mutagenic primer. The oligonucleotide. . .

DETDESC:

DETD(120)

To make the **Factor** **IX**/VII expression construction, 1 ug of pD2 was digested at 37.degree. C. for 1 hour with 20 units of Bam HI. . .

DETDESC:

DETD(127)

Expression of **Factor** **IX**

DETDESC:

DETD(128)

Fourteen . . . at 37.degree. C. The DNA . . . obtained to

electrophoresis in 1% agarose and the 1.4 kb band containing the
Factor **IX** sequence was isolated from the gel.

DETDESC:

DETD(121)

The assay for biological activity is based on the ability of **Factor**
IX to reduce the clotting time of plasma from **Factor**
IX-deficient patients to normal. It was done as described by Proctor
and Rapaport (Amer. J. Clin. Path. 36: 212, 1961). Results. . .

DETDESC:

DETD(122)

TABLE 4

Cells/ml	**Factor** **IX** polypeptide (ng/ml)	**Factor** **IX** activity (ng/ml) in	% active protein in
Day (.times.10.sup.-4)	supernatant	pellet	supernatant. . .

DETDESC:

DETD(123)

The amount of **Factor** **IX** polypeptide was determined by ELISA
essentially as described in Example 6 using polyclonal rabbit antisera to
Factor **IX**. Following the incubation of the wells with the
Factor **IX**-containing samples, the wells were rinsed and incubated
1 hour at room temperature with 200 ul of affinity purified rabbit
polyclonal anti-**Factor** **IX** conjugated to alkaline phosphatase
diluted 1:1000 in PBS containing 1% BSA and 0.05% Tween 20. The wells are
then rinsed. . .

DETDESC:

DETD(124)

As shown in Table 4, 70%-80% of the **Factor** **IX** polypeptide is
secreted into the media, and about 50% of this is biologically active. No
Factor **IX** activity was detected in the cell pellets.

DETDESC:

DETD(125)

Several additional analyses were performed to demonstrate that the cells
were secreting authentic **Factor** **IX**. Samples containing **Factor**
IX activity according to the above assay were incubated with Factor
VIII-deficient plasma but did not affect the clotting time, indicating
that the activity was due to authentic **Factor** **IX** rather than a
non-specific clotting agent. This conclusion was further verified by
depletion of **Factor** **IX** activity from the samples with a specific
antibody. Ninety-seven to ninety-eight percent of the **Factor** **IX**
activity was immunoprecipitated from cell supernatants with a rabbit
polyclonal antibody against **Factor** **IX**. This antibody also
precipitated over 90% of the **Factor** **IX** activity from normal
plasma. No **Factor** **IX** activity was removed from the supernatant
[illegible]

SETDESC:

SETD(139)

An . . . coding region joined to the partial Factor VII cDNA was constructed. The vector, designated pM710C, was generated by inserting the **Factor** **IX** leader-5' Factor VII sequence from pM711C and the 3' Factor VII sequence from FIX/VII/pD2 into plasmid pD3, which comprises the. . .

SETDESC:

SETD(146)

Expression . . . replicative form of pM711C was digested with Bam HI and Xba I and the 552 base pair fragment comprising the **Factor** **IX** leader and 5' Factor VII sequence was gel purified. Plasmid FIX/VII/pD2 was digested with Xba I and Bam HI and. . .

CLAIMS:

CLMS(6)

6. . . . of Factor VII, the joined sequences coding for a protein which upon activation has substantially the same biological activity for **blood** **coagulation** as Factor VIIa.

CLAIMS:

CLMS(7)

7. . . . of Factor VII, the joined sequence coding for a protein which upon activation has substantially the same biological activity for **blood** **coagulation** as Factor VIIa.

CLAIMS:

CLMS(14)

14. . . . of Factor VII, the joined sequences coding for a protein which upon activation has substantially the same biological activity for **blood** **coagulation** as Factor VIIa, the joined sequences being followed downstream by a polyadenylation signal.

CLAIMS:

CLMS(15)

15. . . . of Factor VII, the joined sequences coding for a protein which upon activation has substantially the same biological activity for **blood** **coagulation** as Factor VIIa, the joined sequences being followed downstream by a polyadenylation signal.

CLAIMS:

CLMS(22)

22. . . . of Factor VII, the joined sequences coding for a protein which upon activation has substantially the same biological activity for **blood** **coagulation** as Factor VIIa, the joined sequences being followed downstream by a polyadenylation signal.

CLAIMS:

22. . . . of Factor VII, the joined sequences coding for a protein which upon activation has substantially the same biological activity fo **blood** **coagulation** as Factor VIIa, the joined sequences being followed downstream by a polyadenylation signal.

CLAIMC:

CLMS(24)

24. A method for producing a protein having biological activity for **blood** **coagulation** mediated by Factor VIIa, comprising: establishing a mammalian host cell which contains a DNA construct comprising a DNA sequence encoding. . .

CLAIMS:

CLMS(31)

31. . . . of Factor VII, the joined sequences coding for a protein which upon activation has substantially the same biological activity for **blood** **coagulation** as Factor VIIa.

CLAIMS:

CLMS(32)

32. . . . of Factor VII, the joined sequences coding for a protein which upon activation has substantially the same biological activity for **blood** **coagulation** as Factor VIIa.

=> ab bit

US PAT NO: 4,414,334 L14: 1 of 1

ABSTRACT:
Removal of ambient oxygen from aqueous liquids is effectively catalyzed by enzymatic deoxygenation systems comprising alcohol oxidase in the presence of alcohol optionally with catalase. Suitable deoxygenation systems described can be used to alleviate corrosion and oxidative degradation in areas such as oil field fluids, circulating water systems, water storage tanks, alcoholic beverages and foodstuffs. As desired, the enzymatic systems can be immobilized on supports or used in solution.

1. 4,414,334, Nov. 8, 1983, Oxygen scavenging with enzymes; Donald C. Hitzman, 435/252; 422/7, 12; 435/190, 291, 938

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(FILE 'USPAT' ENTERED AT 11:14:10 ON 03 JAN 02)

L1	9	S	GAMMA-CARBOXYLATION
L2	928	S	BLOOD(W)COAGULATION
L3	928	S	BLOOD-COAGULATION
L4	6	S	L1 AND L2
L5	185	S	FACTOR-IX
L6	5	S	L4 AND L5
L7	33	S	METHYLOTROPHIC
L8	259	S	HANSENULA
L9	10	S	L7 AND L8
L10	14	S	METHANOL-OXIDASE
L11	2	S	L9 AND L10
L12	3	S	L7 AND L10
L13	200	S	PICHIA
L14	1	S	L10 AND L13

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